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(54) Title: PROSTATE-SPECIFIC MEMBRANE ANTIGENS AND METHODS OF MAKING AND USING (57) Abstract A purified and isolated human PSM' protein which is substantially free of other human proteins is provided. The N-terminal amino acid sequence of the PSM' protein is two amino acids short compared to the N-terminal amino acid sequence of the putative human PSM' protein. Methods of purifying the PSM' protein, antibodies against the PSM' protein of the present invention are also provided. Immunoassays and immunoassay kits are also provided for detecting prostate cancer in a patient.		

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5 **Prostate-specific Membrane Antigens and Methods of Making and Using**

Background of the Invention

Field of the Invention

10 The invention relates generally to prostate cancer related proteins and specifically to the identification and purification of an alternatively spliced variant of prostate-specific membrane antigen (PSMA) called PSM' antigen. In another aspect, it relates to antibodies which recognize such antigens. In yet another aspect, it relates to methods for producing such antibodies and diagnostic uses therefor.

Description of the Prior Art

15 Throughout this application, various references are referenced within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

20 Prostate cancer is the second leading cause of cancer death in men (1), being especially prevalent among men over the age of 50. There are approximately 73,000 new cases of prostate carcinoma each year, and approximately 23,000 deaths per year. The importance of early diagnosis is readily apparent by the statistic that the five-year survival rate in patients with localized prostate tumors is twice as high as those with
25 disseminated cancer. Additionally, the detection of recurrences prior to the development of subjective symptoms or clinical manifestations of the disease, and the monitoring and evaluation of therapy, are clearly important to an improved prognosis. The identification of reliable prostate cancer markers, therefore, contributes significantly to early diagnosis as well as staging, assessing and monitoring the disease.

30 Several biomarkers have been used for screening, diagnosis, and predicting disease progression (2, 3). Examples of the biomarkers include prostate specific antigen (PSA), and prostatic acid phosphatase (PAP). The relative amount of PSA and/or PAP in prostatic cancer is reduced as compared to normal or benign tissue.

5 Therefore, the measurement of serum PSA is suggested as a potential screening method for prostatic cancer.

A newer marker of prostate cancer, prostate specific membrane antigen (PSMA) was originally identified in LNCaP human prostatic adenocarcinoma cells by its immunoprecipitation with the monoclonal antibody (MAb), 7E11-C5 (4). The Mab
10 7E11-C5 was developed by immunizing mice with the membrane fraction of LNCaP human prostatic adenocarcinoma cells. Studies on PSMA in serum have suggested that its expression may be linked to a more aggressive clinical phenotype (5).

Immunohistochemical studies have identified PSMA in normal prostate, benign prostatic hypertrophy (BPH), prostate cancer and kidney (4). Low levels of the
15 protein have also been reported in the small intestine, colon (6) and the capillary endothelium of a variety of tumors (6, 7). A radioimmunoconjugate of the 7E11-C5 antibody designated CYT356 is currently being used as an imaging agent for prostate cancer (8).

The cDNA coding for PSMA was obtained from a LNCaP cDNA library (9).
20 It coded for a putative type II transmembrane protein consisting of a short intracellular segment (amino acids 1-18), a transmembrane domain (amino acids 19-43) and an extensive extracellular domain (amino acids 44-750). The extracellular domain contains a region with 54% homology to the transferrin receptor.

Recently, an alternatively spliced variant of PSMA RNA called PSM' was
25 described (10). PSMA and PSM' cDNAs are identical except for a 266-nucleotide region near the 5' end of PSMA cDNA (nucleotides 114-380) that is absent from PSM'. The absent region includes the translation initiation codon and codons for the putative transmembrane domain of PSMA. The protein product of PSM' would therefore lack the transmembrane domain of PSMA as well the signal sequence of
30 PSMA. The location of the protein in the cell would probably be cytoplasmic because of the omission of the transmembrane domain.

By examining the expression of PSMA and PSM' mRNA using RNASE protection assays, it was discovered that in LNCaP human prostatic cancer cells and in

5 primary prostate tumors, PSMA is the dominant form. In normal human prostate,
however, more PSM' is expressed than PSMA. Benign prostatic hypertrophy samples
showed about equal expression of both variants (10). For example, the ratio of
PSMA:PSM' level (also called a tumor index) ranges from 9-11 in LNCaP, from 3-6
10 in carcinoma of the prostate, from 0.75-1.6 in benign prostatic hypertrophy, and from
0.075-0.45 in a normal prostate. The index reflects the increased expression of PSMA
over PSM' following the progression from normal to tumor state. This tumor index
may be a useful indicator for the measurement of tumor progression.

Thus, it is important to determine if the putative PSM' protein exists, and to
characterize the protein in order to determine its roles in tumor progression. It is also
15 desirable to develop an effective and specific assay for monitoring tumor progression.

5 Summary of the Invention

It is an object of the present invention to develop a method for identifying and isolating PSM' protein, and to provide a substantially purified PSM' protein. It is also an objective of the present invention to provide an effective and specific assay for monitoring tumor progression.

10 In accordance with one aspect of the present invention, provided is a purified and isolated human PSM' protein which is substantially free of other human proteins. The N-terminal amino acid sequence of the PSM' protein is two amino acids short compared to the N-terminal amino acid sequence of the putative human PSM' protein.

15 The present invention also provides a method of producing human PSM' from a cell line containing both PSMA and PSM'. The method includes the steps of: (a) preparing a cell lysate from the cell line, (b) removing PSMA from the cell lysate, (c) absorbing PSM' from the cell lysate onto a solid phase bound to a monoclonal antibody specific for PSMA and PSM', and (d) recovering PSM' from the solid phase.

20 In accordance with another aspect of the present invention, provided is a monoclonal antibody or a fragment thereof recognizing the PSM' protein of the present invention. Also provided is a polyclonal antibody that recognizes the PSM' protein of the present invention.

The present invention also provides a monoclonal antibody or a fragment thereof recognizing both PSM' and PSMA.

25 The present invention further provides a method of making a monoclonal antibody that is specific for PSM' protein. In accordance with one embodiment of the present invention, the method comprises the steps of:

- (a) synthesizing a peptide composed of the first 15 to 20 amino acids of PSM' protein with an added Cys at the C-terminus,
- 30 (b) conjugating the peptide to a carrier through the C-terminal Cys,
- (c) immunizing a mouse or a suitable host with the conjugates of step (b),
- (d) fusing spleen cells of the immunized mouse or other suitable host with suitable myeloma cells, thereby obtaining a mixture of hybrid cell lines,

- 5 (e) culturing the hybrid cell lines in a suitable medium,
- (f) selecting and cloning the hybrid cell lines producing a monoclonal antibody recognizing the PSM' antigen, and
- (g) recovering the monoclonal antibody produced thereby.

In accordance with a further aspect of the present invention, provided is an immunoassay for determining the PSM' in a sample. One embodiment of the present invention provides an immunoassay comprising the steps of:

- 10 (a) providing a monoclonal antibody which is specific to PSM',
- (b) contacting the monoclonal antibody with the sample under a condition that the monoclonal antibody binds to the PSM' of the sample,
- 15 (c) measuring the amount of bound monoclonal antibody, and
- (d) relating the measured amount of bound monoclonal antibody to the amount of PSM' in the sample.

Yet another aspect of the present invention provides a method for determining PSM' in a sample which contains both PSM' and PSMA. In accordance with one embodiment of the present invention, the method comprises the steps of:

- 20 (a) providing a first monoclonal antibody which recognizes both PSM' and PSMA;
- (b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to either the PSM' or the PSMA of the sample,
- 25 (c) measuring the amount of the bound first monoclonal antibody, contacting the monoclonal antibody with the sample under a condition that the monoclonal antibody binds to the PSM' of the sample,
- (d) providing a second monoclonal antibody which recognizes only PSMA,
- 30 not PSM',
- (e) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,
- (f) measuring the amount of the bound second monoclonal antibody, and

5 (g) determining the amount of PSM' by subtracting the amount of bound second monoclonal antibody from the amount of the bound first monoclonal antibody, and relating the subtracted amount of monoclonal antibody to the amount of PSM'.

A further aspect of the present invention provides a method for predicting prostate cancer progression. In accordance with one embodiment of the present
10 invention, the method comprises the steps of:

(a) providing a first monoclonal antibody which recognizes both PSM' and PSMA,

(b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to either the PSM' or the PSMA of
15 the sample,

(c) measuring the amount of bound first monoclonal antibody, contacting the monoclonal antibody with the sample under a condition that the monoclonal antibody binds to the PSM' of the sample,

(d) relating the measured amount of the bound first monoclonal antibody to
20 the amount of PSM' and PSMA in the sample.

(e) providing a second monoclonal antibody which recognizes only PSMA, not PSM',

(f) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,

25 (g) measuring the amount of bound second monoclonal antibody,

(h) relating the measured amount of bound second monoclonal antibody to the amount of PSMA in the sample, and

(i) determining the ratio of the amount of PSMA to the amount of PSM' and PSMA.

30 In one embodiment of the present invention, a method for predicting prostate cancer progression in a sample containing both PSM' and PSMA by using antibodies of the present invention that are specific for PSM' protein includes the steps of:

(a) providing a first monoclonal antibody specific for PSM' ,

- 5 (b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to the PSM' of the sample,
- (c) measuring the amount of bound first monoclonal antibody,
- (d) relating the measured amount of bound first monoclonal antibody to the amount of PSM' in the sample,
- 10 (e) providing a second monoclonal antibody specific to PSMA,
- (f) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA
- (g) measuring the amount of bound second monoclonal antibody,
- (h) relating the measured amount of bound second monoclonal antibody to
- 15 the amount of PSMA in the sample, and
- (i) determining the ratio of the amount of PSMA to the amount of PSM' and PSMA.

 Yet another aspect of the present invention provides a kit for determining prostate cancer progression. In accordance with one embodiment, the kit includes

20 carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing a monoclonal antibody that recognizes PSM'. In one embodiment, the kit includes another container containing a monoclonal antibody that recognizes PSMA.

 One aspect of the present invention provides a method for the detection of

25 cancer in a suspected cancer patient. In accordance with one embodiment of the present invention, the method includes contacting a tissue specimen obtained from the patient with an antibody recognizing PSM', and determining the sites on the specimen to which the antibody is bound by immunohistochemical means.

 Another aspect of the present invention provides a method for the *in vivo*

30 diagnosis of prostate cancer in a suspected cancer patient. In accordance with one embodiment of the present invention, the method comprise administering a predetermined diagnostic effective amount of an antibody recognizing the PSM' of the present invention, and detecting the sites of localization of the antibody, the antibody

5 'being administered in a pharmaceutically acceptable carrier and labeled so as to permit detection.

10 A further aspect of the present invention provides a method of treating prostate cancer in a cancer patient comprising administering a predetermined effective amount of an antibody recognizing the PSM' protein of the present invention, the antibody being administered in a pharmaceutically acceptable carrier and conjugated with a suitable therapeutic agent.

The invention is defined in its fullest scope in the appended claims and is described below in its preferred embodiments.

5 Description of the Figures

 The above-mentioned and other features of this invention and the manner of obtaining them will become more apparent, and will be best understood by reference to the following description, taken in conjunction with the accompanying drawings.

 These drawings depict only a typical embodiment of the invention and do not therefore
10 limit its scope. They serve to add specificity and detail, in which:

 FIGURE 1 shows the mapping of PSMA monoclonal antibodies 7E11 and PEQ226 to PSMA and PSM'.

 FIGURE 2 is a western blot showing the reactivity of PSMA Mab PEQ226 with GST-PSMA fusion proteins.¹

15 FIGURE 3 shows the western blots analysis of the enrichment of PSM' from LNCaP cell lysate using immunoaffinity resins.

 FIGURE 4 shows the N-terminal amino acid sequence of PSM' isolated from LNCaP cells.

 FIGURE 5 is a western blot which demonstrates that PSM' is located in the
20 cytoplasm of LNCaP cells.

Detailed Description of the Invention

The present invention provides a purified and isolated human PSM' protein which is substantially free of other human proteins. The PSM' protein is considered substantially free from other human proteins if the protein yields a single major band on a non-reducing polyacrylamide gel. The purity of the PSM' protein can also be determined by amino-terminal amino acid sequence analysis, which analysis is well known in the art.

In accordance with one embodiment of the present invention, the human PSM' protein has an N-terminal sequence as indicated in Figure 4. It is noted that the N-terminal sequence of the PSM' protein differs from the predicted N-terminal sequence of the putative PSM' protein. While the putative translation initiation site for PSM' was identified as residue 58 (Met) of PSMA, the actual N-terminal amino acid by protein sequencing was alanine at residue 60 of PSMA. In addition, it has been observed that PSM' protein resides in the cytoplasm of a cell.

The human PSM' protein of the present invention can be prepared in accordance with a method of the present invention. One embodiment of the present invention provides a method of producing human PSM' from a cell line containing both PSMA and PSM'. The method includes the steps of:

- (a) preparing a cell lysate from the cell line,
- (b) removing PSMA from the cell lysate,
- (c) absorbing PSM' from the cell lysate onto a solid phase bound to a monoclonal antibody recognizing PSM'.
- (d) recovering PSM' from the solid phase.

For the purpose of the present invention, a cell line can be any human cell lines that contain both PSMA and PSM' proteins. Examples of such a cell line include, but are not limited to, LNCaP cell line, and the like.² Cell lysates can be prepared from a cell line of the present invention using conventional procedures.

5 In accordance with one embodiment of the present invention, PSMA can be removed from a cell lysate by absorbing PSMA from the cell lysate onto a solid phase bound to a monoclonal antibody specific for PSMA. Examples of monoclonal antibodies specific for PSMA include, but are not limited to, 7E11 monoclonal antibody, and the like.

10 For the purpose of the present invention, monoclonal antibodies recognizing PSM' protein include monoclonal antibodies that are specific for PSM' or for both PSMA and PSM', as long as the monoclonal antibodies recognize PSM'. The term "specific for PSM'" as used herein means that the monoclonal antibody only recognizes PSM', not PSMA. The term "specific for both PSM' and PSMA" as used herein means
15 that the monoclonal antibody can recognize both PSM' and PSMA.

One aspect of the present invention provides antibodies or fragments thereof that are specific for both PSMA and PSM'. Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibodies preparations are provided. Examples of monoclonal antibodies
20 that recognize both PSMA and PSM' include, but are not limited to, PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.³ In accordance with one embodiment of the present invention, to purify PSM' protein, one uses monoclonal antibody PEQ226, generated by hybrid cell line ATCC Deposit #HB 9131, deposited June 24, 1986.

25 Monoclonal antibodies that recognize both PSMA and PSM' proteins can be generated by methods known to one skilled in the art (Kohler, et al., Nature, 256:495, 1975). For the purpose of the present invention, antigens that may be used to generate the monoclonal antibodies that recognize both PSMA and PSM' include, but are not limited to, plasma membranes from LNCaP cells or from prostate cancer patients,
30 PSMA protein, PSMA protein fused to a carrier protein such as complete Freund's adjuvant (CFA), PSMA synthetic peptides and PSMA fusion proteins.

5 Another aspect of the present invention provides an antibody or a fragment thereof that binds to the PSM' protein of the present invention, but not to PSMA proteins. Antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibodies preparations are provided.

10 The term antibody as used in this invention is meant to include intact molecules as well as fragments thereof, such as, but not limited to, Fab, Fab', F(ab')₂, and Fv etc., which are capable of binding an epitopic determinant on a PSMA or PSM' protein. These antibody fragments retain some ability to selectively bind with their antigens or receptors and are defined as follows:

- 15 (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of the whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain;
- 20 (2) Fab', the fragment of an antibody molecule, can be obtained by treating the whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;
- 25 (3) (Fab')₂, the fragment of an antibody, can be obtained by treating the whole antibody with the enzyme pepsin without subsequent reduction; F(ab')₂ is a dimer of two Fab' fragments held together by two disulfide bonds;
- (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains, can be obtained by genetical engineering methods; and
- 30 (5) Single chain antibody ("SCA"), defined as a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule, can be obtained by genetical engineering methods.

5 Methods of making these fragments are known in the art. (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1988), incorporated herein by reference).

 As used in this invention, the term "epitope" means any antigenic determinant on an antigen to which the paratope of an antibody binds. Epitopic determinants
10 usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

 Monoclonal antibodies that are specific for PSM' may be made from an antigen containing PSM' protein or fragments thereof by methods well known to those skilled
15 in the art (Kohler, et al., *Nature*, 256:495, 1975). In accordance with one embodiment of the present invention, a synthesized peptide composed of the first 15-20 amino acids of PSM' may be used as an antigen for producing monoclonal antibodies that are specific for PSM'. Preferably, at the C-terminus, Cys is added to the peptide. One example of such synthesized peptides has an amino acid sequence of:

20 *Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys-Phe-Leu-Cys-*

 Accordingly, in one embodiment of the present invention, a mouse or other suitable host is immunized with the synthesized peptides of the present invention. In one embodiment, the peptides are conjugated to a carrier protein, such as KLH, prior to immunization. Following immunization, the spleen cells of the immunized mouse
25 are fused with the cells from a suitable mouse myeloma line to obtain a mixture of hybrid cell lines. The hybrid cell lines are cultured in a suitable medium and, thereafter, hybrid cell lines producing an antibody having a specific reactivity with the PSM' protein of the present invention are selected and cloned, and the monoclonal antibodies thus produced are recovered.

30 In accordance with one embodiment of the present invention, a method of making monoclonal antibodies of the present invention also includes a step of screening for monoclonal antibodies that recognize PSM', but not PSMA. In accordance with one embodiment of the present invention, to select a monoclonal

antibody that is specific for PSM', not PSMA, antibodies produced by hybrid cell lines will be tested on an identical peptide that is used as the antigen. Those antibodies that are tested positive will also be tested on the sample peptide but with the peptide's N-terminus alanine blocked with His-Asn-Met. For example, the amino acid sequence of the peptide with a blocked N-terminus is:

*His-Asn-Met-Ala-Phe-Lou-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys--
Phe-Leu-Cys*

Clones that fail to detect the N-terminus blocked peptide but are able to detect the PSM' peptide are saved and tested on PSMA and PSM' proteins to confirm their specificity to the PSM' protein, not the PSMA protein.

For the purpose of the present invention, monoclonal antibodies can be bound to many different solid phase carriers. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetites. In one embodiment of the present invention, insoluble carriers are used such as a microtiter plate. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

The monoclonal antibodies of the present invention that are specific for PSM' can be used in any subject in which it is desirable to administer *in vitro*, or *in vivo* immunodiagnosis or immunotherapy. Accordingly, the present invention also provides methods for *in vitro* expression in human samples, particularly in patients with prostate carcinoma. In accordance with the present invention, PSM' expression can be detected in patient tissue samples by immunohistochemical and/or in patient fluid samples by *in vitro* immunoassay procedures. A determination of the expression of PSM' in patient specimens is of significant diagnostic utility and may be indicative of or correlate with the progression of a disease state.

Immunohistochemical methods for the detection of antigens in patient tissue specimens are well known to the art, and need not be described in detail herein. For example, methods for the immunohistochemical detection of antigens are generally

5 described in Taylor, Arch. Pathol. Lab. Med. 102:113 (1978). Briefly, in the context of the present invention, a tissue specimen obtained from a patient suspected of having prostate cancer is contacted with an antibody, preferably a monoclonal antibody, specific for the PSM' protein. The site at which the antibody is bound is thereafter determined by selective staining of the tissue specimen by standard
10 immunohistochemical procedures.

Similarly, the general methods of the in vitro detection of antigenic substances in patient fluid samples by immunoassay procedures are also well known in the art and require no repetition herein. For example, immunoassay procedures are generally described in Paterson et al., Int. J. Can. 37:659 (1986) and Burchell et al., Int. J. Can.
15 34:763 (1984). According to one embodiment of the present invention, an immunoassay for determining the PSM' protein in a sample, comprising the steps of:

- (a) providing a monoclonal antibody which is specific to PSM',
- (b) contacting the monoclonal antibody with the sample under a condition that the monoclonal antibody binds to the PSM' of the sample,
- 20 (c) measuring the amount of bound monoclonal antibody, and
- (d) relating the measured amount of bound monoclonal antibody to the amount of PSM' in the sample.

For the purpose of the present invention, any body fluid that may contain the PSM' protein may be used in the immunoassay of the present invention. Examples of
25 body fluid samples include, but are not limited to, urine, saliva, serum and semen.⁴

The antibody used in the immunoassay in accordance with the present invention, can be any antibody that is specific to the PSM' protein. Both monoclonal antibodies and polyclonal antibodies may be used as long as such antibodies possess the requisite specificity for the antigen provided by the present invention. Preferably,
30 monoclonal antibodies are used.

5 In accordance with another embodiment of the present invention, antibodies that recognize both PSM' and PSMA may also be used in an immunoassay for determining PSM' in a biological fluid sample. In accordance with this embodiment of the present invention, a method for determining the PSM' protein in a sample which contains both PSM' and PSMA includes the steps of:

10 (a) providing a first monoclonal antibody which recognizes both PSM' and PSMA,

(b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to either the PSM' or the PSMA of the sample,

15 (c) measuring the amount of bound first monoclonal antibody,

(d) providing a second monoclonal antibody which recognizes only PSMA, not PSM',

(e) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,

20 (f) measuring the amount of the bound second monoclonal antibody, and

(g) determining the amount of PSM' by subtracting the amount of the bound second monoclonal antibody from the amount of the bound first monoclonal antibody, and relating the subtracted amount of the monoclonal antibody to the amount of PSM'.

25 In one embodiment of the present invention, monoclonal antibodies that recognize both PSMA and PSM' include, but are not limited to, PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086. The monoclonal antibody that recognizes only PSMA, not PSM', may be monoclonal antibody 7E11.

30 Antibodies of the present invention may also be used in an *in vitro* immunoassay to predict cancer progression, particularly prostate cancer progression. Accordingly, one embodiment of the present invention provides a method for predicting prostate cancer progression in a sample containing both PSM' and PSMA by

5 using antibodies of the present invention that are specific for PSM' protein. The method includes the steps of :

- (a) providing a first monoclonal antibody specific for PSM' ,
- (b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to the PSM' of the sample,
- 10 (c) measuring the amount of the bound first monoclonal antibody,
- (d) relating the measured amount of the bound first monoclonal antibody to the amount of PSM' in the sample,
- (e) providing a second monoclonal antibody specific to PSMA,
- (f) contacting the second monoclonal antibody with the sample under a
- 15 condition that the second monoclonal antibody binds to PSMA,
- (g) measuring the amount of the bound second monoclonal antibody,
- (h) relating the measured amount of bound second monoclonal antibody to the amount of PSMA in the sample, and
- (i) determining the ratio of the amount of PSMA to the amount of PSM'
- 20 and PSMA.

Another embodiment of the present invention provides a method for predicting prostate cancer progression in a sample containing both PSM' and PSMA by using antibodies of the present invention that are specific to both PSM' and PSMA proteins. The method includes the steps of :

- 25 (a) providing a first monoclonal antibody which recognizes both PSM' and PSMA,
- (b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to either the PSM' or the PSMA of the sample,
- 30 (c) measuring the amount of the bound first monoclonal antibody, contacting the monoclonal antibody with the sample under a condition that the monoclonal antibody binds to the PSM' of the sample,

5 (d) relating the measured amount of the bound first monoclonal antibody to the amount of PSM' and PSMA in the sample,

(e) providing a second monoclonal antibody which recognizes only PSMA, not PSM',

10 (f) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,

(g) measuring the amount of the bound second monoclonal antibody,

(h) relating the measured amount of the bound second monoclonal antibody to the amount of PSMA in the sample, and

15 (i) determining the ratio of the amount of PSMA to the amount of PSM' and PSMA.

The term "specific for PSMA" as used herein means that the monoclonal antibody only recognizes PSMA, not PSM'. As discussed above, the ratio of PSMA to PSM' in a sample can be a useful indicator for the measurement of tumor progression since there is an increased expression of PSMA over PSM' in a prostate cancer patient following the progression from normal to tumor state.

20 For the purpose of the present invention, monoclonal antibodies can be utilized in liquid phase or bound to a solid phase carrier. Monoclonal antibodies can be bound to many different carriers and used to determine the PSM' protein contained in a sample. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetites. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Examples of insoluble carriers include, but are not limited to, a bead, and a microtiter plate. Those skilled in the art will know of other suitable carriers for binding monoclonal antibodies, or will be able to ascertain such using routine experimentation.

30 In addition, the monoclonal antibodies in these immunoassays can be detectably labeled in various ways. For example, the monoclonal antibodies of the present invention can be coupled to low molecular weight haptens. These haptens can then be

5 specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin, which reacts with avidin, or dinitrophenyl, pyridoxal, and fluorescein, which can react with specific anti-hapten antibodies.

Furthermore, monoclonal antibodies of the present invention can also be coupled with a detectable label such as an enzyme, radioactive isotope, fluorescent
10 compound or metal, chemiluminescent compound or bioluminescent compound. The binding of these labels to the desired molecule can be done using standard techniques common to those of ordinary skill in the art.

One of the ways in which the antibodies can be detectably labeled is by linking them to an enzyme. This enzyme, in turn, when later exposed to its substrate will react
15 with the substrate in such a manner as to produce a chemical moiety which can be detected by, for example, spectrophotometric or fluorometric means (ELISA system). Examples of enzymes that can be used as detectable labels are horseradish peroxidase, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate
20 isomerase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase. For increased sensitivity in the ELISA system, the procedures described can be modified using a biotinylated antibody reacting with avidin-
peroxidase conjugates.

25 The amount of antigen can also be determined by labeling the antibody with a radioactive isotope. The presence of the radioactive isotope would then be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful are ^3H , ^{125}I , ^{123}I , ^{32}P , ^{35}S , ^{14}C , ^{51}Cr , ^{36}Cl , ^{57}Co , ^{58}Co , ^{59}Fe , ^{75}Se , ^{111}In , $^{99\text{m}}\text{Tc}$, ^{67}Ga , and ^{90}Y .

30 Determination of the antigen is also possible by labeling the antibody with a fluorescent compound. When the fluorescently labeled molecule is exposed to light of the proper wavelength, its presence can then be detected due to fluorescence of the dye. Among the most important fluorescent labeling compounds are fluorescein

5 isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine.

Fluorescence emitting metal atoms such as Eu (europium), and other lanthanides, can also be used. These can be attached to the desired molecule by means of metal-chelating groups, such as DTPA or EDTA.

10 Another way in which the antibody can be detectably labeled is by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoglobulin is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, aromatic acridinium
15 ester, imidazole, acridinium salt, and oxalate ester.

Likewise, a bioluminescent compound may also be used as a label. Bioluminescence is a special type of chemiluminescence which is found in biological systems and in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent molecule would be
20 determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase, and aequorin.

Qualitative and/or quantitative determinations of the PSM' in a sample may be accomplished by competitive or non-competitive immunoassay procedures in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay
25 (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the present invention can be done utilizing immunoassays which are run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. Those of skill in the art will know, or can readily discern, other immunoassay formats without undue
30 experimentation.

The term "immunometric assay" or "sandwich immunoassay" includes a simultaneous sandwich, forward sandwich and reverse sandwich immunoassay. These terms are well understood by those skilled in the art. Those of skill will also appreciate

5 that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

The present invention also provides for the *in vivo* diagnosis and therapy of cancer in humans, particularly of prostate carcinoma. Methods for *in vivo* tumor
10 imaging and therapy are generally described in Holt S. et al., Can. Med. Assoc. J. 129:18 (1983) and Sfakianakis, G. et al., J. Nucl. Med. 23:840 (1982). In accordance with the present invention, methods for tumor localization and detection may be performed by administering to a suspected cancer patient a predetermined
15 diagnostically effective amount of an antibody recognizing the targeted tumor-associated proteins, such as PSM' or PSMA, and thereafter, detecting the sites of localization of the antibody by standard imaging techniques.

The antibodies, preferably monoclonal antibodies used for the purpose of *in vivo* diagnosis are labeled so as to permit detection. Examples of the types of labels and labeling techniques are described above and require no repetition here. Preferably,
20 antibodies are labeled with a radio nuclide emitting gamma-radiation, and administered to the patient in a pharmaceutically acceptable carrier, e.g., buffered saline and human serum albumin.

In using the monoclonal antibodies of the invention for the *in vivo* detection of antigens, the detectably labeled antibody is given a dose which is diagnostically
25 effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the targeted antigens for which the monoclonal antibodies are specific.

The concentration of detectably labeled monoclonal antibodies which is
30 administered should be sufficient such that the binding to those cells having the targeted antigens is detectable compared to the background. Further, it is desirable that the detectably labeled monoclonal antibody be rapidly cleared from the circulatory system in order to give the best target-to-background signal ratio.

5 As a rule, the dosage of the detectably labeled monoclonal antibody for *in vivo* diagnosis will vary depending on such factors as age, sex, and the extent of disease of the individual. Such dosages may vary, for example, depending on whether multiple injections are given, the degree of antigenic burden, and other factors known to those of skill in the art.

10 For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important factor in selecting a radioisotope for *in vivo* diagnosis is that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a large number of photons in the
15 140-250 keV range, which may readily be detected by conventional gamma cameras.

In accordance with methods permitted by the present invention for cancer therapy, a predetermined effective amount of an antibody, preferably a monoclonal antibody, recognizing the tumor-associated antigens characterized by the invention, is
20 administered to a cancer patient. The antibody is conjugated with a suitable therapeutic agent, e.g., radioisotopes, preferably emitters of beta particles, drugs, toxins, or biological proteins selected for delivery to the tumor site, and administered to the cancer patient in a pharmaceutically acceptable carrier, e.g., buffered saline and human serum albumin.

25 In the context of *in vivo* cancer therapy, those skilled in the art will appreciate that the "predetermined effective amount" of antibody suitable for use may vary with individual patients and will depend upon such factors as the disease state, the circulating antigen concentration, antibody specificity, antibody kinetics and biodistribution, and whether an antibody is administered alone or as an antibody
30 cocktail. Antibody preparations comprising mixtures of antibodies or fragments thereof, i.e., antibody cocktails, having specificity for the tumor-associated antigens of the invention may be used in certain instances to enhance the detection, localization and treatment of tumors. Accordingly, as used herein, the term "antibody" includes

5 fragments thereof such as Fab, Fab¹ and Fab² fragments or mixtures thereof including mixtures with whole antibodies.

One aspect of the present invention provides a kit for determining PSM' in a biological sample. The kit includes carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing a
10 monoclonal antibody that recognizes PSM' in a sample. In one embodiment of the present invention, the monoclonal antibody recognizes only the PSM' protein. In another embodiment of the present invention, the kit contains two containers, wherein one container contains a first monoclonal antibody recognizing both PSM' and PSMA, and the another container contains a second monoclonal antibody recognizing only
15 PSMA. Examples of the first monoclonal antibody include, but are not limited to, PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086. One example of the second monoclonal antibody is 7E11.

The following examples are intended to illustrate, but not to limit, the scope of the invention. While such examples are typical of those that might be used, other
20 procedures known to those skilled in the art may alternatively be utilized. Indeed, those of ordinary skill in the art can readily envision and produce further embodiments, based on the teachings herein, without undue experimentation.

EXAMPLE I

Monoclonal Antibodies That Recognize both PSM' and PSMA

1. Monoclonal antibodies that recognize only PSMA, not PSM'

The 7E11 antibody was obtained from Dr. Gerry Murphy, Pacific Northwest Cancer Foundation, Seattle, WA. Its development and clinical use with ¹¹¹In⁵ has been described previously (4). 7E11 recognizes the N-terminus portion of PSMA
30 (residues 1-7) (13).

2. Monoclonal antibodies that recognize both PSMA and PSM'

5 To develop monoclonal antibodies that recognize both PSMA and PSM', monoclonal antibodies to various regions of PSMA are first developed. The monoclonal antibodies to PSMA are then selected for their ability to recognize the PSM' of the present invention.

10 In order to optimize the development of monoclonal antibodies (Mabs) to various regions of PSMA, a variety of immunization approaches was utilized. The PM2M Mabs were obtained from A/J mice (Jackson Labs) which received the following immunization protocol. On day 1 the mice were injected i.p. with 50 ug of plasma membranes purified from LNCaP cells with alum. Two weeks later the mice received a second injection of 25 ug of membranes with alum. Those mice which
15 responded with good titers were injected i.p. 3 weeks later with 10 ug of PSMA purified from LNCaP cells with alum. The mice were finally boosted with 10 ug of purified PSMA (i.v.) 3 days prior to fusion. The mice were sacrificed and their spleens removed. Cell fusion was carried out according to the procedure of Kohler and Milstein, Nature 256, 4495-497 (1975). 1×10^8 splenocytes were fused in 1.0 ml of a
20 fusion medium composed of 35% polyethylene glycol (PEG 1500) in an APMEM medium (Flow Laboratories, Inglewood, California) with 2.5×10^7 P3.653 myeloma cells. Following fusion, cells were cultured in a HAT medium (hypoxanthine, aminopterin, thymidine) at 37°C in a humidified 5% CO₂ incubator. Antibodies produced by hybridomas were screened by an enzyme-linked immunoabsorbent binding
25 assay (ELISA) on biotinylated purified PSMA absorbed to avidin coated microtiter plates. Those clones producing signals with OD >0.5 were selected for expansion. Clones were also saved based on their reactivity with LNCaP plasma membranes and lack of reactivity with DU145 plasma membranes.

30 The PM2H and PM2J Mabs were obtained from Balb/C mice (Harlan Labs) which received the following immunization schedule. On day 1 the mice were injected i.p. with 25 ug PSMA purified from LNCaP cells with alum. Two weeks later the mice received a second injection of 10 ug purified PSMA with alum. The mice were finally boosted i.v. with 10 ug of purified PSMA three days prior to fusion. Fusions

5 were performed as described above. These fusions were screened the same way the PM2M fusion was screened (see above).

PM1X310.5 was obtained from an A/J mouse which received 50 ug of T7-134-437 PSMA fusion protein i.p. with CFA. This was followed two weeks later with 25 ug of fusion protein injected i.p. with IFA. A final boost of fusion protein (25 ug i.v.)
10 was given three days prior to fusion. The clone was selected based on its strong reactivity with T7-134-437 PSMA fusion protein as well as LNCaP membranes and minimal reactivity on DU145 membranes.

PMIT485.5 was obtained from an A/J mouse which received 50 ug of T7-438-750 PSMA fusion protein i.p. with alum. This was followed two weeks later with 25
15 ug of fusion protein injected i.p. with alum. After a third injection of fusion protein (25 ug) the mice were bled and good titers were obtained on T7-438-750 PSMA. The mice were finally boosted with 25 ug fusion protein i.v. 3 days prior to fusion. The clone was selected based on its strong reactivity with T7-438-750 PSMA as well as LNCaP membranes and lack of reactivity with DU145 membranes. The PM2B clones
20 were developed in a similar manner except that the fusion protein, GST-438-750 PSMA was used as the immunogen.

Monoclonal antibody PEQ226 was developed from a Balb/C mouse which was immunized with tumor plasma membranes obtained from an autopsy specimen of prostate carcinoma. The mice were injected 5 times i.p. at 14 day intervals with 200
25 ug of prostate carcinoma plasma membranes. Three days after the 5th immunization, a mouse was sacrificed and the spleen was harvested. The fusion technique has been previously described. Hybridomas were selected based on their binding to prostate carcinoma membranes and failure to bind to normal liver membranes by ELISA. PEQ226 was more recently shown to bind to purified PSMA as well as recombinant
30 PSMA.

Purification and Identification of PSMA from LNCaP Cells.

Cells were harvested from 20 T160 flasks by mild trypsinization. The cells were lysed by the addition of 5 volumes of 1% NP-40, 150 mM NaCl in 25 mM Tris-

5 HCl pH 7.4 (extraction buffer) at 4 C. This mixture was stirred overnight (16-20 h) at 4 C. The solution was centrifuged at 19,000 x g for 1 h. The supernatant solution was removed and filtered with 0.2 micron filter. This clarified solution was then incubated with 0.5 mls affinity resin consisting of the 7E11 Mab covalently bound to a matrix support at a concentration of 6 mg 7E11 per ml of resin. After gentle shaking
10 overnight, the resin/supernatant solution was centrifuged on a tabletop centrifuge to sediment the beads. The beads were then washed 4X with 10 volumes (5mls) of extraction buffer. The beads were transferred to a column with a fritted support and washed 5X with 10 column volumes of extraction buffer. The beads were then washed 5X with 10 column volumes of 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% reduced
15 Triton X-100. The PSMA protein was eluted with 100 mM glycine, 150 mM NaCl, 1% reduced Triton X-100, pH 2.5. The fractions were immediately neutralized by the addition of 1/10 volume 1 M Tris pH 8.0.

The eluent was concentrated and applied to a 4-20% polyacrylamide gel. A major band at 100 kDa was seen with coomassie blue stain. This band was cut out of
20 the gel and digested with trypsin. The peptide fragments were eluted and applied to reverse phase chromatography. The peaks were collected and N-terminally sequenced. Several peaks showed sequences consistent with internally cleaved fragments of PSMA.

Mammalian transient expression of PSMA

25 Full-length PSMA cDNA was isolated from the plasmid PDR2 (Seattle) and was cloned into the plasmid pCDNA3 (Invitrogen) to yield the clones pCDNA3-PSMA#7. DNA sequencing was used to verify the identity and proper orientation of the insert. This clone was transfected into COS-1 cells using lipofectamine (Life Technologies) and the transient expression of PSMA was monitored by western blot
30 using the 7E11 monoclonal antibody. Results showed that the PSMA (100 kDa) protein was expressed in the cell lysates 24-48 hours after transfection.

Preparation of Plasma Membranes

5 Approximately 5×10^8 LNCaP cells or DU145 cells (ATCC, Rockville, MD) were harvested from tissue culture flasks by mild trypsinization. The cells were centrifuged at 100x g for 5 min at 4 °C and then diluted 5-fold in a homogenization buffer (30 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM tris-HCl pH 7.2) and
10 disrupted by nitrogen cavitation (500 PSI for 20 min). All subsequent steps were performed at 4 °C. The cell lysate was centrifuged at 1,000 x g for 5 min and the supernatant was removed and centrifuged at 100,000 x g for 1 h. The supernatant (cytosol fraction) was removed and the pellet was resuspended in a 1.0 ml homogenization buffer with freshly added PMSF and layered onto an ultracentrifuge
15 tube containing 16 mls of 40% sucrose and 16 mls of 20% sucrose in a homogenization buffer. The gradient was spun in a Beckman L8 ultracentrifuge at 100,000 x g for 16 h. The turbid layer at the 20%/40% interface was collected, suspended in a homogenization buffer with fresh PMSF added and centrifuged at
20 100,000 x g for 1 h. The membrane fraction (pellet) was collected and resuspended in a minimal volume (0.5-1.0 ml) homogenization buffer. Protein was quantitated using the BCA assay (Pierce, Rockville, 11).

Cloning and Purification of Fusion Proteins

 Various fragments of PSMA were amplified by PCR. PACGHISNTA-PSMA1.9 (Seattle) was used as the template to amplify fragments PSMA 134-437 and
25 PSMA 438-750. PDR2 plasmid (Seattle) was used as the template to amplify PSMA 1-173 fragments. These fragments were cloned into PGEX (Promega) or pET5a (Novagen) vectors to generate GST and T7 PSMA fusion proteins, respectively. The DNA of the resulting fusion proteins were transformed into E. coli BL21 (DE3) (for T7 fusion proteins) or E. coli DH 5a (for GST fusion proteins). Single cell clones
30 were propagated in an LB broth and protein production was induced by adding IPTG (0.4 mM). The cell pellets were collected after 2 hours of induction and resuspended in a sample buffer for analysis. The PSMA 134-437 and 438-750 fusion protein induced cell pellets were lysed by sonication and the pellet containing the inclusion

5 bodies was collected and fusion proteins were purified by HPLC. For PSMA 1-173, the E. coli pellet was resuspended in a sample buffer and used without further purification

Cell Fractionation

10 Plasma membrane and cytosol fractions were prepared according to a previously described and published procedure (12) with minor modifications. Approximately 5×10^8 LNCaP cells were harvested from tissue culture flasks by mild trypsinization. The cells were centrifuged at $100 \times g$ for 5 min at 4 C and then diluted 5-fold in a homogenization buffer (30 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM tris-HCl pH 7.2) and disrupted by nitrogen cavitation (500 PSI for 20 min). All
15 subsequent steps were performed at 4 C. The cell lysate was centrifuged at $1,000 \times g$ for 5 min and the supernatant was removed and centrifuged at $100,000 \times g$ for 1 h. The supernatant (cytosol fraction) was removed and the pellet was resuspended in a 1.0 ml homogenization buffer with freshly added PMSF (1 mM) and layered onto an ultracentrifuge tube containing 16 mls of 40% sucrose and 16 mls of 20% sucrose in a
20 homogenization buffer. The gradient was spun in a Beckman L8 ultracentrifuge at $100,000 \times g$ for 16 h. The turbid layer at the 20%/40% interface was collected, suspended in homogenization buffer with fresh PMSF added and centrifuged at $100,000 \times g$ for 1 h. The membrane fraction (pellet) was collected and resuspended in a minimal volume (0.5-1.0 ml) homogenization buffer. Protein was quantitated using
25 the BCA assay (Pierce, Rockville, IL).

5

Western Blot Analysis

Samples were reduced and denatured and electrophoresed on 4-20% polyacrylamide gels and blotted onto nitrocellulose. Blots were incubated with primary Mabs followed by incubation with goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, Pa). Antibody reactivity was detected using the enhanced chemiluminescence (ECL) reagent (Amersham, Arlington Heights, Il).

10

Table 1 summarizes monoclonal antibodies obtained based on the methods described above.

15

Table 1⁶

PSMA MABs

Antibody	Isotype	Immunogen	ELISA (OD 490) PSMA	ELISA (OD 490) LNCaP Membranes	ELISA (OD 490) DU145 Membranes
PM1T485.5	G1	T7438-750	4.1	3.2	0.4
PM1X310.5	G1	T7134-437	3.9	5.4	0.1
PM2B110.5	G2b	T7438-750	2.5	2.3	0
PM2B181.3	G2b	GST438-750	2.6	2.4	0
PM2E086	G1	PSMA	3.1	1.8	0.4
PM2E343	G1	PSMA	8.4	3.5	0.1
PM2E346	G1	PSMA	1.6	0.4	0
PM2E522	G1	PSMA	3.3	0.8	0
PM2H043	G1	PSMA	4.9	2.9	0
PM2H207	G1	PSMA	3.8	4.4	8
PM2H164	G1	PSMA	1.4	0.8	0
PM2H327	G1	PSMA	3.3	1	0
PM2H015	G1	PSMA	1.65	1	0.1

PM2H112	G1	PSMA	2.4	1	0
PM2J001	G1	PSMA	6.9	1.9	0
PM2J004	G1	PSMA	8.8	3.8	0
PM2J010	G1	PSMA	2.6	1.7	0.2
PM2H268	G1	PSMA	7.2	2.2	0
PM2M143	G1	MEMBRANES	2.2	1.1	0.1
PM2M180	G1	MEMBRANES	1.9	1.2	0
PM2M194	G1	MEMBRANES	2.8	1.1	0.1
PM2M396	G1	MEMBRANES	2.5	0.8	0
PM2M440	G1	MEMBRANES	5.4	4.1	0.1
PM2M474	G1	MEMBRANES	4.9	3.3	0
PM2M515	G1	MEMBRANES	1.1	0.6	0
PM2M092	G1	MEMBRANES	2.3	0.9	0.1
PM2M528	G1	MEMBRANES	2.1	0.7	0
PM2M015	G1	MEMBRANES	4.1	4.7	0.9
PM2M345	G1	MEMBRANES	5.3	6.1	4.9
PM2M217	G1	MEMBRANES	2.2	0.6	0
PM2M104	G1	MEMBRANES	3.4	1.4	0.2
7E11	G1	MEMBRANES	3.1	1.5	0
PEQ226	G1	MEMBRANES	1.5	1.5	0
PEE447	G1	MEMBRANES	4.1	2.1	6.2

5

Antibodies that are positive to PSMA are further tested against PSM' protein of the present invention by using routine immunoassays. The results are summarized in table 2.

5

Table 2
PSM' Monoclonal Antibodies

Antibody	Isotype	Immunogen	Detection of PSM' by Western Blot
PM1T485.5	G1	T7 438-750	positive
PM2H043	G1	PSMA	positive
PM2H207	G1	PSMA	positive
PEQ226	G1	membranes	positive
PM1X310.5	G1	T7 134-437	positive
PM2B110.5	G2b	T7 438-750	weak
PM2H327	G1	PSMA	weak
PM2B181	G2b	GST 438-750	weak
PM2H164	G1	PSMA	weak
PM2H327	G1	PSMA	weak
PM2E343	G1	PSMA	positive
PM2E086	G1	PSMA	positive
7E11	G1	membranes	negative

10

The results indicate that monoclonal antibodies that are immunoreactive to both PSM' and PSMA include PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

15

EXAMPLE II

Purification and Sequencing of PSM'

A two-step immunoaffinity chromatography procedure was utilized to purify PSM' from a crude lysate of the human prostate carcinoma cell line, LNCaP. The first

5 MAb (7E11) has previously been shown to bind to the N-terminus of PSMA (13). However, since the PSM' cDNA is missing the 266-nucleotide region at the 5' end of PSMA (10), it was speculated that 7E11 would not bind PSM'. The second monoclonal antibody utilized in the present invention was PSMA MAb, PEQ226, which potentially would bind to both PSMA and PSM' (see Fig 1).

10 Figure 1 shows the mapping of PSMA MAb 7E11 and PEQ226 to PSMA and PSM'. The schematic of full-length PSMA and alternatively spliced PSM' showing the transmembrane domain (TM) and transferrin receptor homologous region (TR). Binding regions for 7E11 and PEQ226 are indicated by arrows. Figure 2 is a western blot showing the reactivity of PSMA Mabs, 7E11 (lanes 1,3,5,7) and PEQ226 (lanes 15 2,4,6,8) with GST-PSMA fusion proteins. Lanes 1-2, GST-PSMA 1173; Lanes 3-4, GST-PSMA 134-437, Lanes 5-6, GST-PSMA 438-750; Lanes 7-8, recombinant PSMA. Figure 2 shows that PEQ226 binds to a T7 fusion protein of PSMA which spans the 134-437 domain of the protein.

Both antibodies 7E11 and PEQ226 were used to purify the PSM' of the present invention. They were bound to agarose beads (AminoLink resin, Pierce, Rockford, IL) and utilized in tandem to first remove full length PSMA from the lysate (7E11-agarose beads) and then capture the remaining PSM' on the second antibody resin (PEQ226-agarose beads). The details of the experiment are described below.

25 LNCaP cells obtained from American Type Culture Collection (Rockville, Maryland) were grown in RPMI media with 10% horse serum (Gibco, Grand Island, NY) and maintained in 5% CO₂ at 37 C. Cells were grown in a T160 flask until 80% cell confluency was obtained. Cells were lysed following the addition of 2 mls of 1% triton X-100, 10% glycerol, 15 mM MgCl₂, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium vanadate, 10 uM aprotinin, 50 mM Hepes pH 7.5. The crude lysate was 30 incubated with 1.0 ml of 7E11-AminoLink beads prepared according to manufacturer's instructions (Pierce, Rockford, Illinois) for 14 h at 4 C. The unbound fraction was then incubated with 1 ml of PEQ226-AminoLink beads for 4 h at 4 C. Beads were washed five times with 10 mls of 1% triton x-100, 10% glycerol, 15 mM MgCl, 50

5 mM Hepes pH 7.5 and then eluted with 1 ml of 2% sodium dodecyl sulfate in 10 mM sodium phosphate, 150 mM NaCl pH 7.5. The eluents and unbound fractions were western blotted and probed with both the 7E11 and PEQ226 Mabs. The eluent from the PEQ226-AminoLink beads was blotted to a PVDF membrane and stained with coomassie blue. The band was cut out of the blot and sequenced on a Procise Protein
10 Sequencer (ABI, Foster City, CA).

The western blot of the eluted proteins and unbound fractions from both columns with 7E11 and PEQ226 is shown in Figure 3. Figure 3 shows enrichment of PSM' from LNCaP cell lysate using immunoaffinity resins. Two sequential immunoaffinity resins were utilized to enrich specifically for PSM'. LNCaP cell lysate
15 was incubated with 0.5 mls 7E11-aminoLink resin. The unbound fraction was incubated with PEQ226-aminoLink resin. The eluted fractions and unbound fractions were examined on western blots with antibodies at 10ug/ml. Lane 1, 3 ul of 7E11-resin eluent detected with 7E11; Lane 2, 3 ul of PEQ226-resin eluent detected with 7E11; Lane 3, 3 ul of 7E11-resin eluent detected with PEQ226; Lane 4, 3 ul of
20 PEQ226-resin eluent detected with PEQ226; Lane 5, 6 ul of the 7E11-resin flow-through fraction detected with 7E11; Lane 6, 6 ul of the 226-resin flow-through fraction detected with 7E11; Lane 7, 6 ul of the 7E11-resin flow-through fraction detected with PEQ226; Lane 8, 6 ul of the 226-resin flow-through detected with PEQ226. Figure 3 shows that the protein which was eluted from the PEQ226 column
25 contained a molecule of approximately 95kDa which was detected with PEQ226 (lane 4) but not with 7E11 (lane 2). This protein migrated slightly further than the full length PSMA which was eluted from the 7E11-agarose beads (lanes 1 and 3).

In order to confirm the identity of the PEQ226-reactive protein, the entire PEQ226 eluent was concentrated and loaded in a single lane and blotted to PVDF
30 paper. The blot was stained with coomassie blue and the 95kDa band was cut out and sequenced. The sequence matched the predicted protein sequence for PSM' deduced from the cDNA sequence. The only deviation from the predicted sequence was the absence of residues 58 (Met) and 59 (Lys) at the N-terminus. While the putative

5 translation initiation site for PSM' was identified at residue 58 (Met), the actual N-terminal amino acid by protein sequencing was alanine at residue 60 of PSMA. The N-terminus sequence of PSM' is shown in Figure 4. Unlike full length PSMA from LNCaP cells, PSM' was not N-terminally blocked.

10 This result indicates that the two column procedure is quite effective in first depleting the lysate of the majority of full length PSMA and then capturing the PSM' on the second column. The PSM' protein migrates slightly further down the gel as expected since it is slightly smaller (59 amino acids shorter than PSMA). The band on western blot is also wider than PSMA. This may be due to a greater degree of differential glycosylation of the PSM' protein. Monoclonal antibodies reactive with
15 other epitopes on PSM' also produce this broad band staining (data not shown). PSM' has 25 potential phosphorylation sites, 10 N-myristoylation sites and 9 N-glycosylation sites (Su SL, Huang I, Fair WR, Powell CT, Heston WD, "Alternatively spliced variants of prostate-specific membrane antigen RNA: Ratio of expression as a potential measurement of progression" Cancer Res 55: 1441-1443 1995.).

20 EXPERIMENT III

Localization of the PSM' in the cytoplasm of LNCaP cells.

Since PSM' lacks the transmembrane domain expressed by PSMA, PSM' may reside in the cytoplasm of the cell. In order to test this hypothesis, cytosol and plasma
25 membrane preparations of LNCaP cells were prepared by differential sucrose gradient centrifugation and western blotted both fractions as well as whole cell lysate (Fig 5). According to Figure 5, LNCaP crude cell lysate (lanes 1 and 4), LNCaP cytoplasm, (lanes 2 and 5) and LNCaP plasma membranes (lanes 3 and 6) were loaded at 0.5ug/lane and probed with the PSMA specific Mab, 7E11 at 10ug/ml (lanes 1-3) or
30 PEQ226 which recognizes both PSMA and PSM' at 10ug/ml (lanes 4-6). The Reactivity in the cytosol fraction was detected only with PEQ226 (lane 4) and not with 7E11 (lane 2). Both antibodies reacted with full length PSMA in the crude cell lysate (lanes 1 and 4) and in the plasma membrane fraction (lanes 3 and 6). The membrane

5 staining in lane 6 with PEQ226 suggests that membranes may contain some PSM' activity. This may be due to minor contamination of the plasma membrane fraction with cytosol or it may represent a subset of PSM' possibly in the process of being secreted from the cell.

PSMA appears to be present in the LNCaP lysate at higher levels than PSM' since PEQ226 only detects the full length 100kDa protein in whole cell lysates (Fig 5
10 lane 4). Longer exposure of this lane did reveal a weaker band comigrating with the PSM' band in lane 5. This result is in agreement with previous evaluations of PSMA and PSM' RNA levels (Su SL, Huang I, Fair WR, Powell CT, Heston WDW. Alternatively spliced variants of prostate-specific membrane antigen RNA: Ratio of
15 expression as a potential measurement of progression. Cancer Res 55: 1441-1443 1995.) which have shown that in LNCaP cells, the ratio of PSMA/PSM' RNA is 9-11.

The present invention is the first study outlining methods to purify PSM' from LNCaP cell lysates which is free of PSMA. N-terminal sequence analysis have confirmed the presence of PSM' and shown that the protein actually begins at Ala-60 in
20 the PSMA sequence. The protein has been shown to reside in the cytoplasm as previously speculated. Ratios of PSMA/PSM' may be of diagnostic value in prostate cancer.

EXAMPLE IV

Method to Produce PSM' Specific Monoclonal Antibodies

25 Immunoaffinity purification of PSM' from LNCaP lysate and N-terminal protein sequencing as described above confirmed earlier RNA sequence data that PSMA and PSM' differ at the N-terminus. Contrary to RNA results which suggested that PSMA, lacks the first 57 amino acids present in PSMA, sequencing of the PSM' protein showed that the first 59 amino acids are missing. Knowledge of the novel N-terminus
30 of PSM', offers a strategy to produce Mabs which bind to PSM' but not to PSMA.

In producing Mabs specific for PSM', one first needs to synthesize a peptide composed of the first 15-20 amino acids of PSM' with an added Cys at the C-terminus. The amino acid sequence of the synthesized PSM' peptide is listed below:

5

*PSM' Peptide**Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys-Phe-Leu-Cys-*

10

The peptide would be conjugated to a carrier Molecule such as KLH or thyroglobulin thru the C-terminal Cys prior to immunization into mice. Following fusion, the resulting hybridomas would be tested for reactivity with the PSM' peptide biotinylated at the C-terminus through the Cys. Unlike the immunogen, this peptide would not be conjugated to a carrier protein. The ELISA screen would involve capture of the biotinylated peptide on streptavidin coated microtiter plates followed by incubation with supernatant from each of the hybridomas wells. Following extensive washing, a secondary goat anti-mouse immunoglobulin conjugated to horseradish peroxidase would be added to each well. Color development would be monitored at OD 490 following the addition of substrate (OPD). Clones which reacted with the PSM' terminus peptide would then be tested on an identical peptide whose N-terminus alanine was blocked with HisAsn-Met. The PSM' N-term blocked peptide is shown below:

20

*PSM' N-term Blocked Peptide**His-Asn-Met-Ala-Phe-Lou-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys-Phe-Leu-Cys*

25

This peptide would also be biotinylated at the C-terminus Cys and then captured on streptavidin coated microtiter plates. Clones that fail to detect the N-terminus blocked peptide but detect PSM' peptide would be saved and tested on LNCAP purified PSMA and PSM' by western blot.

30

The present invention may be embodied in other specific forms without departing from its essential characteristics. The described embodiment is to be considered in all respects only as illustrative and not as restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of the equivalence of the claims are to be embraced within their scope.

5 What is claims is:

1. A purified and isolated human PSM' protein which is substantially free of other human proteins.
2. The purified and isolated human PSM' protein according to claim 1, having a
10 N-terminal amino acid sequence showing in SEQ ID NO.:1.
3. A method of producing human PSM' protein from a cell line containing both PSMA and PSM', comprising the steps of:
 - (a) preparing a cell lysate from the cell line,
 - 15 (b) removing PSMA from the cell lysate,
 - (c) absorbing PSM' from the cell lysate onto a solid phase bound to a monoclonal antibody specific for PSMA and PSM', and
 - (d) recovering PSM' from the solid phase.
- 20 4. The method of claim 3, wherein step (b) further comprising the step of absorbing PSMA from the cell lysate onto a solid phase bound to a monoclonal antibody specific for PSMA.
5. The method of claim 3, wherein the monoclonal antibody is selected from a
25 group consisting of monoclonal antibodies PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.
6. The method of claim 5, wherein the monoclonal antibody is PEQ226.
- 30 7. The method of claim 6, wherein the monoclonal antibody PEQ226 is produced by a hybridoma cell line deposited with the ATCC under ATCC Accession No. HB 9131.

- 5 8. The method of claim 3, wherein the cell line is LNCaP cell line.
9. A monoclonal antibody or a fragment thereof immunoreactive with the PSM' protein of claim 1.
- 10 10. The monoclonal antibody or a fragment thereof of claim 9 being of murine origin.
11. The monoclonal antibody of claim 9 being specific for PSM' protein of claim 1.
- 15 12. The monoclonal antibody of claim 9 being immunoreactive with human PSMA protein.
13. The monoclonal antibody of claim 12 being selected from a group consisting of monoclonal antibodies PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5,
20 PM2E343, and PM2E086.
14. A hybridoma cell line that secretes the monoclonal antibody of claim 9.
15. The hybridoma cell line of claim 14 being of a murine origin.
- 25 16. A hybridoma cell line that secretes the monoclonal antibody of claim 13.
17. The hybridoma cell line of claim 16 being of a murine origin.
- 30 18. A Polyclonal antibody immunoreactive with the PSM' protein of claim 1.
19. A method of making a monoclonal antibody that is immunoreactive with PSM' protein of claim 1, the method comprising the steps of

- 5 (a) synthesizing a peptide composed of the first 15 to 20 amino acids of PSM' protein with an added Cys at the C-terminus,
- (b) conjugating the peptide to a carrier through the C-terminal Cys,
- (c) immunizing a mouse or a suitable host with the conjugates of step (b),
- (d) fusing spleen cells of the immunized mouse or other suitable host with
- 10 suitable myeloma cells, thereby obtaining a mixture of hybrid cell lines,
- (e) culturing the hybrid cell lines in a suitable medium,
- (f) selecting and cloning the hybrid cell lines producing monoclonal antibodies recognizing the PSM' antigen, and
- (g) recovering monoclonal antibodies produced thereby.

15 20. The method of claim 19 further comprising a step of screening for monoclonal antibodies that recognize the PSM' protein, but not PSMA protein.

20 21. The method of claim 20, wherein the screening step includes testing the monoclonal antibodies produced by hybrid cell lines of step (e) on the peptide of step (a), and testing the same monoclonal antibodies on the peptide of step (a) with its N-terminus blocked.

25 22. The method of claim 21, wherein the peptide of step (a) has an amino acid sequence of

Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys-Phe-Leu-Cys-

30 23. The method of claim 22, wherein the N-terminus of the peptide of step (a) is blocked with His-Asn-Met, and whereby the amino acid sequence of the N-terminus blocked peptide is

His-Asn-Met-Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys-Lys--Phe-Leu-Cys.

- 5 24. A monoclonal antibody prepared by the method of claim 19.
25. A monoclonal antibody prepared by the method of claim 20.
26. A monoclonal antibody prepared by the method of claim 23.
- 10 27. An immunoassay for determining the PSM' protein contained in a sample,
comprising the steps of
- (a) providing a monoclonal antibody which is specific to the PSM' protein,
 - (b) contacting the monoclonal antibody with the sample under a condition

15 that the monoclonal antibody binds to the PSM' of the sample,

 - (c) measuring the amount of bound monoclonal antibody, and
 - (d) relating the measured amount of bound monoclonal antibody to the
amount of PSM' in the sample.
- 20 28. The immunoassay of claim 27, wherein the sample is selected from a group
consisting of urine, saliva, and semen.
29. An immunoassay for determining the PSM' protein contained in a sample which
contains both PSM' and PSMA proteins, the immunoassay comprising the steps of:
- 25 (a) providing a first monoclonal antibody which recognizes both PSM' and
PSMA,
- (b) contacting the first monoclonal antibody with the sample under a
condition that the first monoclonal antibody binds to either the PSM' or the PSMA of
the sample,

30 (c) measuring the amount of bound first monoclonal antibody,

 - (d) providing a second monoclonal antibody which recognizes only PSMA,
not PSM',

5 (e) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,
(f) measuring the amount of bound second monoclonal antibody, and
(g) determining the amount of PSM' by subtracting the amount of bound second monoclonal antibody from the amount of the bound first monoclonal antibody,
10 and relating the subtracted amount of monoclonal antibody to the amount of PSM'.

30. The immunoassay of claim 29, wherein the first monoclonal antibody is selected from a group consisting of monoclonal antibodies PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

15 31. The immunoassay of claim 29, wherein the second monoclonal antibody is 7E11.

32. A method for predicting prostate cancer progression in a sample containing
20 both PSM' and PSMA by using antibodies of the present invention that are specific for PSM' protein, the method comprising the steps of:

(a) providing a first monoclonal antibody specific for PSM',
(b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to the PSM' of the sample,
25 (c) measuring the amount of bound first monoclonal antibody,
(d) relating the measured amount of bound first monoclonal antibody to the amount of PSM' in the sample,
(e) providing a second monoclonal antibody specific to PSMA,
(f) contacting the second monoclonal antibody with the sample under a
30 condition that the second monoclonal antibody binds to PSMA,
(g) measuring the amount of bound second monoclonal antibody,
(h) relating the measured amount of bound second monoclonal antibody to the amount of PSMA in the sample, and

5 (i) determining the ratio of the amount of PSMA to the amount of PSM' and PSMA.

33. A method for predicting prostate cancer progression in a sample containing both PSM' and PSMA by using antibodies of the present invention that are specific to both PSM' and PSMA proteins, the method comprising the steps of :

10 (a) providing a first monoclonal antibody which recognizes both PSM' and PSMA,

(b) contacting the first monoclonal antibody with the sample under a condition that the first monoclonal antibody binds to either the PSM' or the PSMA of the sample,

15 (c) measuring the amount of bound first monoclonal antibody,

(d) relating the measured amount of bound first monoclonal antibody to the amount of PSM' and PSMA in the sample,

(e) providing a second monoclonal antibody which recognizes only PSMA, not PSM',

20 (f) contacting the second monoclonal antibody with the sample under a condition that the second monoclonal antibody binds to PSMA,

(g) measuring the amount of bound second monoclonal antibody,

(h) relating the measured amount of bound second monoclonal antibody to the amount of PSMA in the sample, and

25 (i) determining the ratio of the amount of PSMA to the amount of PSM' and PSMA.

34. The method of claim 33, wherein the first monoclonal antibody is selected from a group consisting of monoclonal antibodies PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

35. The method of claim 33, wherein the second monoclonal antibody is 7E11.

5

36. The method of claims 27, 29, 32, and 33, wherein the monoclonal antibody is detectably labeled.

10

37. The method of claim 36, wherein the bound monoclonal antibody is measured by adding thereto a labeled second antibody with specificity to the monoclonal antibody.

15

38. A kit for determining the prostate cancer progression in a sample comprising carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing a monoclonal antibody that recognizes PSM'.

20

39. The kit of claim 38, wherein the kit comprises another container containing a monoclonal antibody specific for PSMA.

40. The kit of claim 39, wherein the monoclonal antibody that recognizes PSM' is specific for PSM'.

25

41. The kit of claim 39, wherein the monoclonal antibody that recognizes PSM' also recognizes PSMA.

30

42. The kit of claim 41, wherein the monoclonal antibody that recognizes both PSM' and PSMA is selected from a group consisting of PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

43. The kit of claim 38, wherein the monoclonal antibody is detectably labeled.

44. The kit of claim 43, wherein the detectable label is an enzyme or a radiolabel.

5

45. A method for the detection of cancer in a suspected cancer patient comprising contacting a tissue specimen obtained from the patient with an antibody recognizing PSM', and determining the sites on the specimen to which the antibody is bound by immunohistochemical means.

10

46. A method for the *in vivo* diagnosis of prostate cancer in a suspected cancer patient comprising administering a predetermined diagnostic effective amount of an antibody recognizing the PSM' protein of claim 1, and detecting the sites of localization of the antibody, the antibody being administered in a pharmaceutically acceptable carrier and labeled so as to permit detection.

15

47. The method of claim 46, wherein the antibody is a monoclonal antibody.

48. The method of claim 47, wherein the monoclonal antibody is selected from a group consisting of PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

20

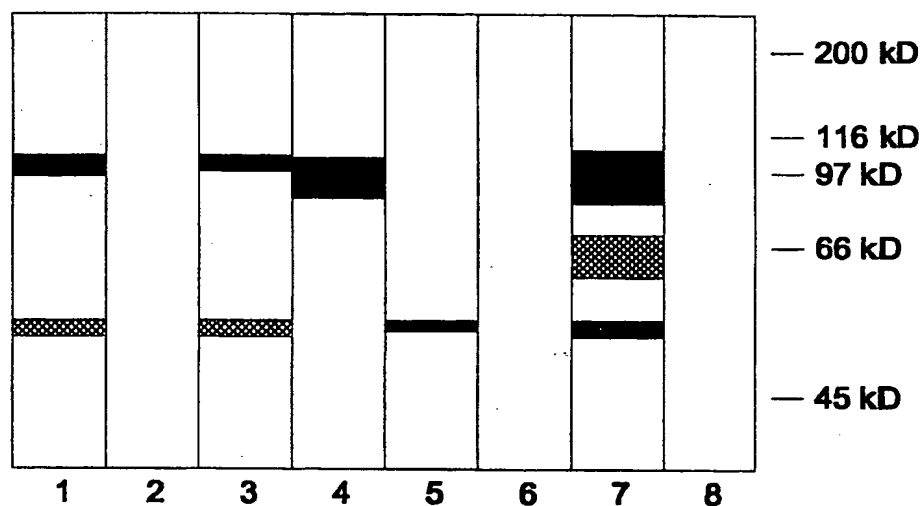
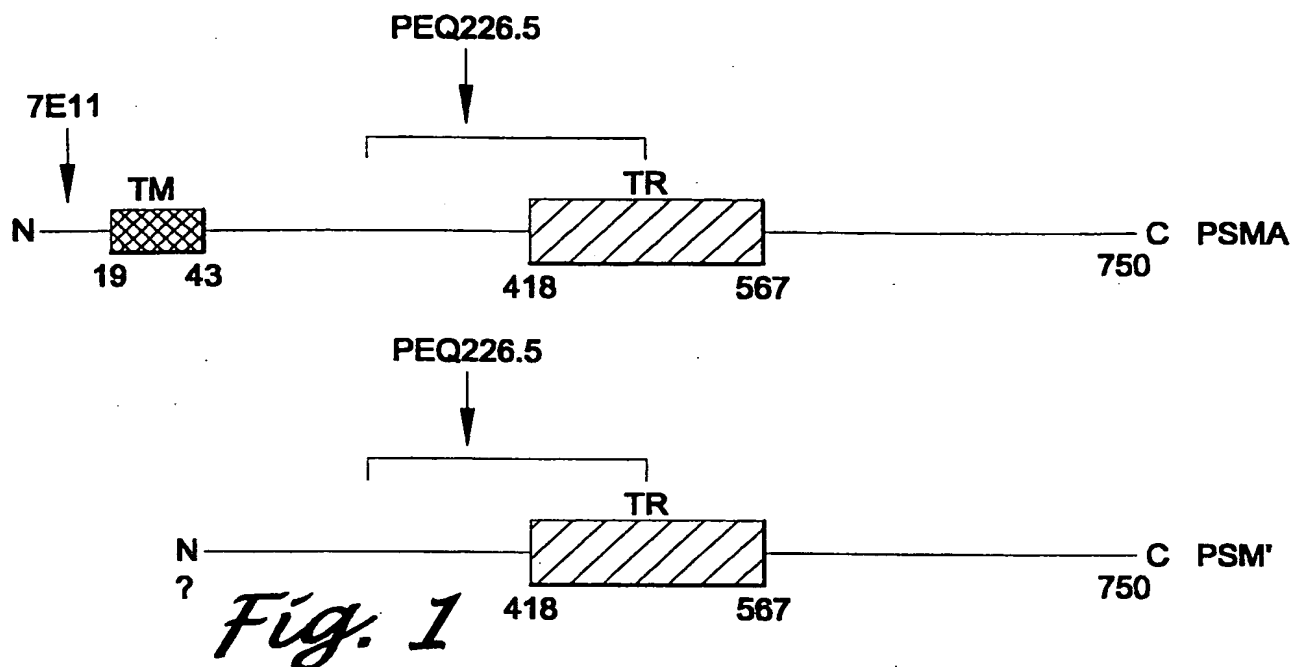
49. A method of treating prostate cancer in a cancer patient comprising administering a predetermined effective amount of an antibody recognizing the PSM' protein of claim 1, the antibody being administered in a pharmaceutically acceptable carrier and conjugated with a suitable therapeutic agent.

25

50. The method of claim 49, wherein the antibody is a monoclonal antibody.

51. The method of claim 50, wherein the monoclonal antibody is selected from a group consisting of PM1T485.5, PM2H043, PM2H207, PEQ226, PM1x310.5, PM2E343, and PM2E086.

30



Ala-Phe-Leu-Asp-Glu-Leu-Lys-Ala-Glu-Asn-Ile-Lys

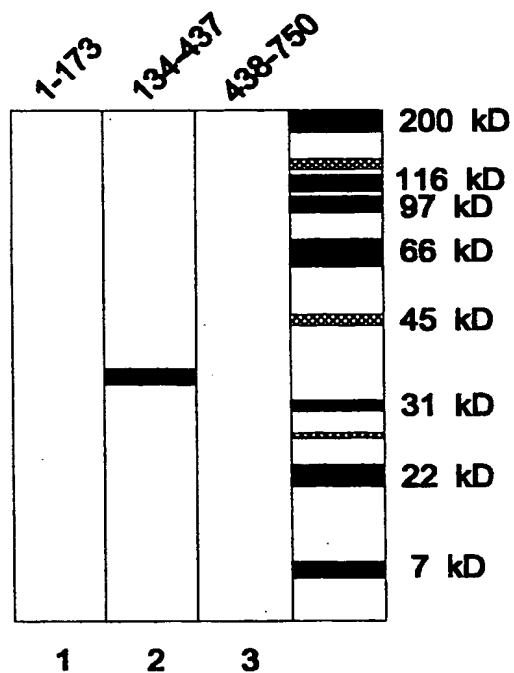
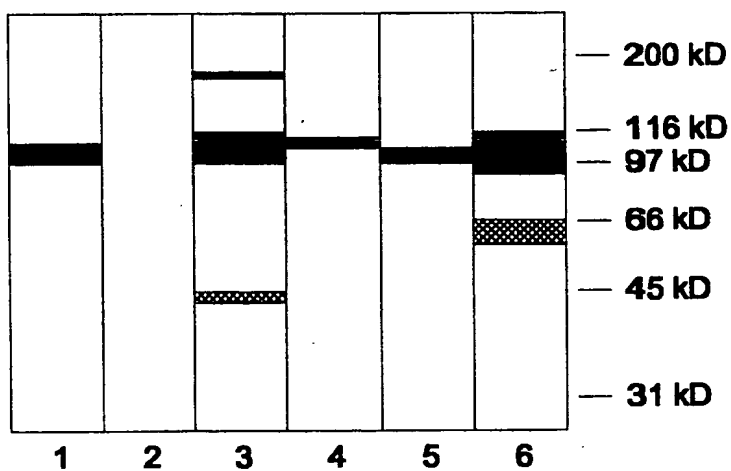
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Fig. 4

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PEQ 226.5 DETECTS FUSION PROTEIN SMA 134-437

*Fig. 2**Fig. 5*

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/03810

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C07K14/705 C07K16/28 C12N5/20 C12N15/06 G01N33/577 G01N33/68 G01N33/574 A61K51/10		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35616 A (PACIFIC NORTHWEST CANCER FOUND) 2 October 1997 (1997-10-02) page 2, line 22 - page 5, line 26 page 20, line 5 - page 26, line 29 ---	1-51
X	WO 96 26272 A (HESTON WARREN D W ; SLOAN KETTERING INST CANCER (US); ISRAELI RON S) 29 August 1996 (1996-08-29) page 3, line 30 - page 4, line 18 page 20, line 1-12 page 23, line 22 - page 24, line 8 page 30, line 27 - page 35, line 14 page 41, line 32 - page 43, line 26 example 6 --- <div style="text-align: center;">-/-</div>	1-51
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
* Special categories of cited documents :		
<div style="display: flex;"> <div style="flex: 1;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">20 July 1999</div>		Date of mailing of the international search report <div style="text-align: center;">28/07/1999</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Covone, M</div>

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte. onal Application No.

PCT/US 99/03810

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ABDEL-NABI H ET AL: "MONOCLONAL ANTIBODIES AND RADIOIMMUNOCONJUGATES IN THE DIAGNOSIS AND TREATMENT OF PROSTATE CANCER"</p> <p>SEMINARS IN UROLOGY, vol. 10, no. 1, 1 February 1992 (1992-02-01), pages 45-54, XP000645163</p> <p>---</p>	9,12-46
A	<p>WO 97 04802 A (PACIFIC NORTHWEST CANCER FOUND ;MURPHY GERALD P (US); BOYNTON ALTO)</p> <p>13 February 1997 (1997-02-13) claim 3 seq.id.22</p> <p>-----</p>	19-23

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 03810

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 46-48, 49-51
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 03810

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 46-48 are directed to a diagnostic method and claims 49-51 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 46-48 49-51

Rule 39.1(iv) PCT - Diagnostic method practised on the human or animal body
Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/03810

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9735616 A	02-10-1997	AU 2555297 A CA 2250141 A EP 0914155 A	17-10-1997 02-10-1997 12-05-1999
WO 9626272 A	29-08-1996	AU 5172596 A CA 2212846 A EP 0812356 A	11-09-1996 29-08-1996 17-12-1997
WO 9704802 A	13-02-1997	US 5788963 A AU 6714696 A EP 0841940 A NZ 315441 A	04-08-1998 26-02-1997 20-05-1998 29-06-1999